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Escherichia coli α-Hemolysin: Characteristics and Probable Role in Pathogenicity

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INTRODUCTION

Escherichia coli is usually considered to be an opportunistic pathogen which constitutes a large portion of the normal intestinal flora of humans. This organism can, however, contaminate, colonize, and subsequently cause infection of extraintestinal sites and is a major cause of septicemia, peritonitis, abscesses, meningitis, and urinary tract infections (UTI) in humans. The source of these *E. coli* pathogens in most cases is believed to be the host's own intestinal flora. Because of its clinical significance *E. coli* has been the subject of numerous investigations in an attempt to define those virulence factors which allow it to initiate and sustain infections. It is now believed that virulence in *E. coli* is multifactorial and that certain properties are associated primarily with virulent strains. One of these is the ability to produce a hemolysin. By far, most *E. coli* cells in the human intestine do not have this capability. Therefore, if the production of infection in extraintestinal sites by intestinal strains is a random phenomenon, most isolates from these infections would also be nonhemolytic. However, a disproportionately high percentage of extraintestinal isolates produce hemolysin, and it therefore has been proposed to be a virulence factor.

The purpose of this review is to present a status report on the *E. coli* hemolysin and to point out those areas where additional research is needed.

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HISTORICAL PERSPECTIVE

Kayser in 1903 reported that some *E. coli* cultures lysed erythrocytes (RBC) (55). He noted that the culture supernatants retained hemolytic activity after being filtered through a Chamberland filter. Dudgeon and Pulvertaft in 1927 also reported hemolytic activity in *E. coli* cultures (19) but could not demonstrate hemolysin in culture filtrates. They assumed that all activity was associated with the *E. coli* cell. Several other reports confirmed the existence of cell-associated hemolytic activity (2, 19, 20, 51, 81, 86). Kayser's report of a filterable hemolysin was confirmed in 1960 when Lovell and Rees (64) obtained a bacteria-free hemolysin preparation by filtration of cultures grown in alkaline meat infusion broth.

Smith in 1963 was the first to clearly differentiate cell-bound and cell-free hemolysin in cultures of *E. coli* grown in alkaline meat extract broth (88). He showed that under the same growth conditions some hemolytic strains of *E. coli* produce cell-free and cell-bound hemolysin simultaneously. Cell-bound hemolysin was not neutralized by antiserum prepared against the cell-free hemolysin, indicating that the two hemolysins might be different. Smith designated the cell-bound hemolytic factor as β-hemolysin and the cell-free factor as α-hemolysin (AH). This was perhaps an unfortunate designation and has caused some confusion since both the α- and β-hemolysins cause β-hemolysis (clear zone of lysis) around colonies on blood agar plates.

Walton and Smith (113) found a third hemolysin (γ-hemolysin) produced by mutants resistant to nalidixic acid. Hemolysin production by these mutants is enhanced by growth in nalidixic acid. Unlike the α- and β-hemolysins, the

γ-hemolysin does not hemolyze human or rabbit RBC but does hemolyze RBC of other species.

Snyder and Koch (94) confirmed the report of Smith (88) and in addition showed that the β- and α-hemolysins were also produced in casein hydrolysate and in a chemically defined medium (CDM). They reported that a heat-stable AH was produced in CDM, whereas a heat-labile hemolysin was found in meat infusion cultures. Muranyi and Juhasz (73) reported that the CDM hemolysin was probably organic acid formed from carbohydrates. However, other work has shown that AH from CDM has a high molecular weight and its hemolytic and cytotoxic activities are destroyed by proteases and lipases (5–7).

Much of the initial work with hemolytic *E. coli* was by investigators in the veterinary sciences. Although an association was made between the presence of hemolytic strains of *E. coli* and the occurrence of enteritis (41) and edema disease (11, 36–41, 56, 87, 96, 100) of swine as well as diseases of other domestic animals (90), there appears to be no direct role for hemolysin in these diseases.

Kayser in 1903 used isolates of hemolytic *E. coli* from abscesses in humans in his studies on AH (55). However, Schmidt in 1909 suggested that the ability of *Bacillus coli-hemolyticus* (*E. coli*) to produce hemolysis did not enhance its pathogenicity (83). Lyon in 1917 implicated hemolytic *E. coli* as a causative agent of cystitis (66). Similarly, Dudgeon et al. (20, 21) in 1921 and 1922 reported hemolysin-producing strains as the causative agents in nearly one-half of *E. coli* UTI. In these studies only 13% of fecal specimens from normal controls contained hemolytic *E. coli*. Numerous other studies described later in this review show an association between hemolytic *E. coli* and infections in humans. The significantly higher association with extraintestinal infections than with normal flora led to the consensus that hemolytic *E. coli* cells are more likely to cause disease than nonhemolytic *E. coli* and, therefore, to studies evaluating the importance of the hemolysin as a virulence factor.

More recent studies have described some of the effects of AH on tissue cells and have proposed a possible role for AH in production of disease. Partially purified preparations of AH from human isolates are cytotoxic for human leukocytes (6, 7) and for fibroblasts in vitro (5). Others report that lysis of RBC may result in making iron available for growth of the bacterium (63, 110). These two effects, i.e., cytotoxicity and stimulation of growth, appear to be the most likely mechanisms by which the hemolysin may function to increase the virulence of *E. coli*.

Transmissible plasmids bearing hemolysin determinants were demonstrated almost two decades ago (91), and more recently hemolytic *E. coli* cells isolated from humans have been shown to carry hemolytic determinants on the chromosome (3, 43, 49, 72, 117). The location and functions of the genes required for hemolysin production and secretion have been investigated. The mechanism whereby AH is produced and excreted appear to be under complex genetic control. Recombinant DNA technology has added to our understanding of the production of the hemolysin and of its potential role in virulence of hemolytic *E. coli*.

PRODUCTION AND SECRETION OF AH

Physiological Aspects

The isolation of hemolytic *E. coli* from a number of clinical sources stimulated interest in the importance of the hemolysin in disease. This led to studies of the conditions and factors required for optimal production and secretion of the hemolysin and the relationship between cell-associated β-hemolysin and cell-free AH.

E. coli AH is produced by growing hemolytic isolates of the bacterium in an alkaline meat extract broth, casein hydrolysate, or a CDM at 37°C (94). AH is produced under both aerobic and anaerobic conditions and in CO_2 (51, 64, 88, 95, 101, 118). Aerobic growth enhances AH production (64, 95). Irrespective of the culture conditions and medium used, both β-hemolysin and AH are produced during the logarithmic phase of growth (84, 88, 94, 95, 97).

Springer and Goebel (97) compared the levels of intracellular hemolysin with the appearance of cell-free hemolysin. Intracellular hemolysin was quantitated by sonically disrupting washed cells and measuring hemolytic activity. Both the intracellular and the cell-free hemolysin pools accumulate up to the midlogarithmic phase of growth, at which time the amount of each declines and reaches a low level as the cells enter the stationary phase. Snyder and Koch (94) showed that both AH and β-hemolysin are produced simultaneously by *E. coli*, with maximum production during logarithmic growth. Production of both declines during the stationary phase of growth. They did not report attempts to disrupt the cells to directly measure internal hemolysin but, like other investigators (85, 88), they could not detect an intracellular form.

Inukai and Kodama (50) suggested that a meat broth component stimulates release of AH from the bacterial cell surface. Jorgensen et al. (53) found that a heat-stable, trypsin-sensitive molecule present in meat broth media is required for AH production and that the amount of AH produced is proportional to the amount of meat factor in the medium. By growing an AH-producing strain of *E. coli* in ^{14}C-labeled glucose and comparing the specific activity of AH with that of the total cellular protein, they showed that <0.33% of the protein present in partially purified AH is of bacterial origin. This suggested that the hemolysin is composed mainly of medium components. The meat broth component and hemolysin are similar in that both are sensitive to trypsin, are acidic, and have similar elution volumes in gel filtration. However, the meat factor is heat stable whereas the hemolysin is heat labile. They concluded that AH may be produced by bacterial modification of a molecule present in meat broth rather than by de novo synthesis by the bacterial cell. An alternative possibility is that AH is produced and secreted in minute amounts and then binds to or aggregates with proteins in complex media.

Additional support for the need of a protein factor in media came from Springer and Goebel (97), who showed that the amount of AH released in an alkaline medium increases 10-fold if the bacterial cells are suspended in meat extract broth or a blood extract broth. They isolated from blood broth a 40,000-dalton basic protein with a pI of 7.5 which gives AH yields similar to those from the meat extract or blood extract broth. This protein was not identified; however, myoglobin and hemoglobin also increase hemolytic activity.

Others found that AH is produced in CDM in the absence of a medium meat factor (5–7, 78, 94). Production of AH in CDM was first described by Snyder and Koch (94). The medium used contains salts, glucose, and ammonium sulfate as a nitrogen source. In addition to glucose, lactose and mannitol also support production of AH, but galactose, mannose, maltose, and sorbitol result in production of only cell-bound hemolysin. In general, carbohydrates that result

in production of AH are those that support better growth of *E. coli*. Using the same CDM, Rennie and Arbuthnott (78) showed that carbohydrates added to starved cultures of *E. coli* result in production of high titers of hemolysin. Again, most carbohydrates which support good bacterial growth also stimulate AH production.

Iron may be important in controlling production of AH. Iron at 30 to 100 μM does not affect hemolysin production in vitro but high concentrations (>100 μM) repress hemolysin production (110). The effect of iron on hemolysin production at concentrations below 30 μM has not been reported. Other studies (63, 110) suggest that a major function of AH in vivo may be to provide iron for growth under iron-limiting conditions. Enhanced growth could result in increased hemolysin production. However, high concentrations of iron would inhibit hemolysin production.

Springer and Goebel (97) described some of the mechanims involved in AH secretion. They found that inhibition of protein synthesis by chloramphenicol results in the accumulation of a limited amount of extracellular hemolysin (AH). This release of hemolysin and its extracellular appearance occur concurrently with the exhaustion of an equivalent amount of intracellular hemolysin and represent final, preformed, periplasmic hemolysin en route through the outer membrane. The release of periplasmic hemolysin from chloramphenicol-treated cells indicates that protein synthesis is not required for excretion. Inhibitors of energy metabolism such as 2,4-dinitrophenol, sodium azide, or potassium cyanide block release of de novo-synthesized hemolysin and result in its intracellular accumulation. None of these inhibitors affects release of preformed periplasmic hemolysin pools, indicating that energy is not required for release of hemolysin through the outer membrane. Processing of the hemolysin protein is required because procaine, a drug which inhibits the processing of exoprotein precursors, blocks hemolysin release into the periplasmic space. Secretion of preformed periplasmic hemolysin pools is inhibited at high pH (8.0) and by low temperatures (0 to 20°C). As a result of these data Springer and Goebel concluded that two transport processes are involved in hemolysin secretion. De novo-synthesized hemolysin is extruded through the cytoplasmic membrane by an energy-dependent process and probably requires processing. The hemolysin accumulates temporarily in the periplasmic space and is then released through the outer membrane by an energy-independent but temperature-dependent process which does not require de novo protein synthesis. These data are supported by other work which shows that multiple cistrons are required for synthesis and secretion of AH (32, 75, 76, 112).

Genetic Aspects

In recent years, the genetic control of *E. coli* AH production and secretion has been studied extensively. It is now apparent that the genes for AH production in most human isolates reside on the bacterial chromosome (3, 43, 49, 72, 117). However, the determinants for AH production by strains isolated from animals usually reside on a plasmid (33, 34, 91). Recombinant DNA, hybridization, and mutagenesis techniques have resulted in an understanding of plasmid incompatability patterns, the location and function of cistrons, the relatedness of AH genes found on plasmids and on the chromosome, and the function of the hemolysin determinants in production and secretion.

General characteristics. Smith and Halls (91) first described the transmissible nature of the hemolytic character in *E. coli*. They found that 10 of 53 hemolytic *E. coli* strains transmitted the hemolytic trait by conjugation with nonhemolytic *E. coli* recipients. The hemolytic phenotype was assumed to be encoded by a plasmid designated Hly. Except for a rare report of colicin transfer (15), Hly is transmitted independently of Col and R factors. Hly can also possess fi^+ (fertility inhibition) characteristics (54, 71, 89, 91, 92) and its introduction into an F^+ recipient often results in the cells becoming resistant to infection by the F-specific bacteriophage MS2.

Transfer of Hly from *E. coli* to other genera also has been shown. Smith and Halls (91) reported that the Hly factor could be transferred to *Salmonella* spp. and some strains of *Shigella* spp. but not to several other species of bacteria. Emody et al. (23) reported the transfer of Hly from *E. coli* to *Proteus morganii* both in vitro and in vivo. Le Minor and Le Coueffic (62) reported transfer of Hly among *E. coli* strains as well as to *Salmonella typhi* and *Salmonella typhimurium*. Introduction of Hly into nonhemolytic *E. coli* results in a relatively stable hemolytic phenotype (91). Hly is less stable in *Shigella* and *Salmonella* spp.

Several investigators studied conditions under which *E. coli* could be cured of the Hly plasmid. Smith and co-workers (91, 93) were unsuccessful in their attempts with acridine orange, ethidium bromide, sodium lauryl sulfate, acriflavine, and UV radiation. However, Mitchell and Kenworthy (70) found that Hly is eliminated at high frequency by the RNA inhibitors of transcription, actinomycin D, rifampin, and streptovaricin. Intercalating DNA inhibitors of replication such as acriflavine, ethidium bromide, daunorubicin, and ethyl violet are less effective. De la Cruz et al. (15) showed differences in sensitivity to rifampin curing among different Hly plasmids. Goebel and Schremph (34) were unable to cure Hly with rifampin.

Smith and Halls (91) speculated that the genes encoding AH (*hly*) in the 43 strains which did not transfer the hemolytic trait are located on the chromosome. Later, Minshew and co-workers (68) also proposed that AH production by some strains of *E. coli* isolated from human extraintestinal infections are probably chromosomally determined. Subsequently, Hull et al. (49) located the hemolysin-encoding locus (*hly*) on the bacterial chromosome near the *ilv* genes in a strain of *E. coli* isolated from the urinary tract.

Plasmid location of hemolysin determinant. Goebel and Schrempf (34) were first to provide direct evidence for plasmid control of hemolysin production in some *E. coli* strains. By sucrose gradient centrifugation and electron microscopy, they demonstrated two large supercoiled circular DNA molecules in hemolytic strains of *E. coli*. Both molecules had characteristics similar to those of other transmissible plasmids and were associated with transfer of hemolysin production to nonhemolytic recipients.

Goebel et al. (33) later showed that some strains of hemolytic *E. coli* possess one or more of three plasmids: plasmid A (65 megadaltons [Mdal]), plasmid B (41 Mdal), and plasmid C (32 Mdal). Upon mating the hemolytic strain of *E. coli*, PM167a, with nonhemolytic strains, recipients of plasmid B only, plasmid C only, plasmids B and C, or plasmids A, B, and C were hemolytic. Recipients of only plasmid A were nonhemolytic. Plasmid A probably possesses an independent transfer factor but not a hemolysin determinant. Hemolytic transconjugants containing only plasmid B or C could transfer hemolytic ability, suggesting that both of these plasmids possessed the hemolysin deter-

TABLE 1. Characteristics of Hly plasmids

Plasmid[a]	Incompatibility grouping	Mol wt ($\times 10^3$)	References[b]
pIP240	FIII	52	61, 62
pSU316	FIII/IV	48	14–16
pSU105	FVI	77	14–16
pSU212	FVI	72	15, 71
pSU1	FVI	77	15, 16
pSU5	Iα/I2	93	14–16
pHly152	I2	41	14, 76
pIP241	I2	42	61, 62
pSU233	?	60	14–16

[a] Most common or recent designation.
[b] pHly52 has also been reported to belong to incompatability group J2 (75).

minant and also independently controlled their own transfer.

Generally, closely related plasmids in the same bacterial cell are unable to coexist. This property, called incompatibility, has been useful for classifying plasmids (10), including those bearing hemolytic determinants, into incompatibility groups (Table 1).

Lawn et al. (60) reported that a hemolytic strain of *E. coli* was sensitive to the F-specific phages MS2 and M13 and was fi^+ and concluded that the Hly plasmid was related to the F incompatibility group of plasmids. Monti-Bragadin et al. (71) also associated the fi^+ character with the Hly plasmid from *E. coli* strain P212. Although cells harboring this plasmid, Hly-P212 (later designated pSU212 [15]), are sensitive to MS2 phage and therefore F^+, this plasmid is compatible with plasmids of all previously known F incompatibility groups (FI through FV). pSU212 was therefore designated as the first member of a new F-like incompatibility group, FVI. Le Minor and associates (61, 62) found that the Hly plasmid pIP240 was also fi^+ and F-like but is a representative of the FIII incompatibility group. Incompatibility of plasmid pSU316 with both type FIII and FIV plasmids (FIII-FIV complex) was reported by De la Cruz et al. (15).

Not all Hly plasmids in *E. coli* are F-like. Royer-Pokora and Goebel (82) reported on two Hly plasmids that show little sequence homology with F or I plasmids. Le Minor and associates (61, 62) demonstrated that plasmid pIP241 is fi^- and incompatible with plasmids in the I2 group. De la Cruz et al. (15, 16) reported that pSU5 is fi^- and belongs to incompatibility group Iα/I2, whereas pSU233, an fi^+ plasmid, was compatible with all F and I groups tested. These reports show that Hly plasmids from *E. coli* are heterogeneous with regard to incompatibility, presumably a direct reflection of DNA content.

De la Cruz et al., (15) made a comprehensive study of Hly plasmids from several representative incompatibility groups. The plasmids varied in molecular mass from 42 to 93 Mdal. Similarities in size were noted only within specific groups. For instance, three FVI plasmids, pSU1, pSU105, and pSU212, have sizes of 77, 77, and 72 Mdal, respectively, whereas the I2 plasmid pIP241 has a molecular mass of 42 Mdal (Table 1). Similarly, electrophoresis of *Hin*dIII restriction digests of the plasmids showed similarities only with plasmids of the same incompatibility group. By inserting Tn*802*, a transposon carrying carbenicillin resistance (Cb^r), into the hly determinant, these investigators created plasmids with a double marker, Cb^r/Hly^-. Introduction of these inactive Hly plasmids into *E. coli* with wild-type plasmids of various incompatibility groups allowed them to follow plasmid segregation and provided direct evidence of the existence of multiple incompatibility groups of Hly plasmids. Reassociation studies with plasmids of various incompatibility groups showed that hemolysin plasmids within individual incompatibility groups, such as FVI (pSU1, pSU105, and pSU202) or FIII-IV (pSU316 and PSU240), share at least 80% sequence homology (16). All F-like plasmids of different incompatibility groups share a 20- to 26-Mdal stretch of common DNA believed to be *traF* (transfer operon).

Royer-Pokora and Goebel (82) used DNA hybridization techniques to detect recombination of Hly plasmids during conjugation. Transconjugants resulting from matings with single-plasmid donors received the plasmids in an unaltered form. When multiple plasmids were present in the donor, extensive recombination occurred. These plasmids are often the same size as the parent plasmids but share decreased sequence homology with the parent plasmid and increased homology with other plasmids in the donor.

De la Cruz et al. (16) reported little or no homology between F-like and I-like plasmids unless both determined AH production. All Hly plasmids, regardless of incompatibility group, share at least a common 5-Mdal region of DNA which presumably contains the hly determinant region. These investigators suggested that the hemolysin determinants of all Hly plasmids are similar regardless of the incompatibility group with which they are associated and that the respective gene products should have similar amino acid composition. Support in favor of this view is that a single antiserum is capable of neutralizing the hemolytic activity of AH from other strains of *E. coli* (88).

De la Cruz et al. (14) showed that *Eco*RI restriction digests of Hly plasmids from five different incompatibility groups contain fragments with the same electrophoretic mobilities as fragments of the Hly plasmid pHly152 (see below; 75, 76). RNA transcribed in vitro from cloned pHly152 restriction fragments (incompatibility group I2) were used as hybridization probes against DNA from pSU105, pSU316, pSU233, and pSU5 (incompatibility groups FVI, FIII/FIV, unclassified, and Iα/I2, respectively). Although some of the plasmids contain very restricted overall sequence homology, a 3.8-Mdal segment of DNA containing the hemolysin determinant was common to all five plasmids.

A possible explanation for the finding of hemolysin determinants on various types of plasmids has recently been proposed. Zabala et al. (120) demonstrated multiple (two to six) copies of a 1.9-kilobase sequence in the Hly plasmids pSU105, pSU233, pSU316, and pHly152, all of which belong to different imcompatibility groups. This common sequence promotes illegitimate recombination, does not encode any phenotypically identifiable proteins, and thus behaves as an insertion element (IS). This IS is larger in size than other *E. coli* IS and unlike other *E. coli* IS does not contain restriction sites for *Pst*I, *Hin*dIII, or *Eco*RI. It is designated as IS-Hly (IS*91* by the Plasmid Reference Center). Diaz-Aroca et al. (18) confirmed by restriction enzyme mapping and hybridization that IS*91* is different from other insertion sequences. IS-Hly is able to promote recombination of Hly plasmids with pACYC184, a plasmid unable to promote its own mobilization. Transfer of the recombined plasmid to recipients resulted in plasmids of three different sizes appearing in transconjugants. These were shown by molecular weight analysis to correspond to (i) pACYC184 plus IS-Hly, (ii) Hly plus pACYC184 plus IS-Hly, and (iii) Hly. The formation of the cointegrate Hly plus pACYC184 plus IS-Hly is assumed to occur in the donor bacterial cell and is promoted by IS-Hly. The hybrid plasmid is stable in *recA* strains but in a $recA^+$ background broke down to two plasmids, Hly and pACYC184 plus IS-Hly. Both plasmids contained the IS.

Plasmid hemolysin determinants and their function. Noegel and associates (75, 76) and Stark and Shuster (98, 99) investigated the location and functions of the genes from pHly152 and pHly185, respectively. Strains harboring these plasmids produce a cell-free extracellular pool of hemolysin (Hly_{ex}) as well as an intracellular pool (Hly_{in}). Two types of nonhemolytic mutants were obtained by chemical and transposon mutagenesis of the plasmids. Some mutants (Hly^+in/Hly^-ex) produce Hly_{in} but cannot export it into the medium, so no Hly_{ex} accumulates. Other mutants (Hly^-in/Hly^-ex) do not synthesize hemolysin and neither Hly_{in} nor Hly_{ex} can be detected.

Restriction fragments from the hemolysin determinant of pHly152 were cloned on a vector plasmid and used to complement transposon (Tn*3*) insertion mutants with defective AH production or export (76). A 3.4-Mdal stretch of plasmid DNA located within three contiguous *Eco*RI fragments (F, L, G) and two *Hin*dIII fragments (E, C) (Fig. 1) contains most of the hemolysin determinant. This region is required for both production and secretion of AH. pHly185 has similar restriction endonuclease sites. At least three cistrons, *cisA*, *-B*, and *-C* (later designated *hlyA*, *-B*, and *-C*) clustered in the hly determinant were found to be involved in synthesis and secretion of AH. Three *hly* cistrons were also found in pHly185 (99). The two cistrons involved in precursor synthesis (*hlyA*) or processing (*hlyC*) or both are on contiguous DNA segments and near a third cistron (*hlyB*) that is required for export of AH. Complementation analysis revealed one difference between pHly185 and pHly152. Expression of pHly185 DNA requires two promoters as contrasted with one for pHly152. Properties other than hemolysin production, such as incompatibility, autonomous replication and copy control, restriction-modification functions, and conjugative transfer, are located within specific restriction fragments outside the Hly region (75).

Early attempts to study the *hlyA* structural gene product were hampered because of the inability to clone the determinants into a high-copy plasmid (75). This suggested that the *hlyA* gene product is toxic to *E. coli* (32, 75). Initially, analysis of gene products from minicells showed that *hlyA* determines a large protein with an estimated molecular weight of 90,000 (76). By insertion of a thermoregulator upstream from *hlyA*, Goebel and Hedgpeth (32) cloned the complete complement of hemolysin genes on the *Bam*HI-*Sal*I and *Hin*dIII E fragments of pHly152 (see Fig. 1) onto a small high-copy plasmid. This recombinant plasmid was designated pANN202-312. They then mapped the hly determinant and determined the functions of the three cistrons *hlyA*, *hlyB*, and *hlyC* (75, 76). The gene product of *hlyA* was found to be a 106,000- to 107,000-dalton nonsecreted cytoplasmic protein which is probably the inactive hemolysin precursor. Recently, Mackman and Holland (67) demonstrated a 107,000-dalton secreted protein in cultures of *E. coli* K-12 containing pHly167 and proposed that it may be the *hlyA* gene product. This protein is secreted from maxicells if the entire pHly167 plasmid is expressed. However, when the hly determinant is introduced into the maxicell on the recombinant plasmid pANN202-312 the protein is located intracellularly, suggesting that the recombinant plasmid lacks some genetic material required for secretion of the 107,000-dalton protein without prior processing.

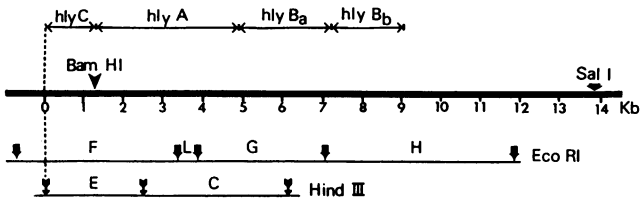

FIG. 1. Physical map of the region on pHly152 containing the four proposed cistrons (↔) of the hly determinant. Restriction endonuclease recognition sites and resulting fragments are shown for *Bam*HI (▼), *Sal*I (◂▸), *Eco*RI (▮), and *Hin*dIII (▮▮). Numbers shown represent length within the determinant in kilobases (kb). These data were compiled from references 32, 75, and 112. Note: Regions containing $hlyB_a$ and $hlyB_b$ are currently incompletely defined.

hlyC codes for a 18,000-dalton protein (45, 76) that appears to be involved in the conversion of the precursor hemolysin to active hemolysin with a proposed molecular weight of 58,000 (45, 76). The *hlyC* gene product is believed to have dual functions of (i) activation and (ii) transport of hemolysin through the cytoplasmic membrane to the periplasm (32, 45, 76).

hlyB is not involved in synthesis of hemolysin but is required for transport of hemolysin from the periplasm to the exterior of the cell (32, 75, 76). Wagner et al. (112) isolated two classes of mutants by mutagenesis of pHly152 with Tn*5*. These mutants are defective either in transport of hemolysin across the outer membrane or in release from the membrane. They accumulate hemolysin in either the periplasmic space or the outer membrane fraction. Two adjacent cistrons ($hlyB_a$ and $hlyB_b$) were identified. $hlyB_a$ is probably responsible for translocation of hemolysin from periplasmic space to the outside of the cell and appears to be transcribed by the same promoter as *hlyC* and *hlyA*. Release of hemolysin from the outer membrane is associated with $hlyB_b$, which has a separate promoter. Cells with a mutation at $hlyB_b$ are phenotypically like those organisms which produce only β- or cell-bound hemolysin. Hartlein et al. (45) showed that the $hlyB_a$ cistron codes for a 46,000-dalton protein located in the outer membrane that binds the hemolysin and transports it through the outer membrane. $hlyB_b$ codes for a protein of 62,000 daltons, most of which is found in the outer membrane, and presumably functions in release of hemolysin from the outer membrane.

Chromosomal hemolysin determinants and their functions. Berger et al. (3) cloned the entire hemolysin region from the chromosome of representative hemolytic strains of *E. coli* from serogroups O4, O6, O18, and O75 into the cosmid pJC74. Each strain carried one copy of the hemolysin determinant per chromosome. The hemolysin determinants of these different strains share considerable homology and have restriction sites similar to those present in plasmids. As with the Hly plasmids, at least three cistrons (A, B, and C) are present on chromosomal hly determinants. Cistron *hlyA* seems to be most variable, whereas *hlyB* and *hlyC* are very conserved. Cistron *hlyC* from plasmid and chromosomal hemolysin determinants are interchangeable and functional hybrids were constructed. Subcloning the *hlyA* and *hlyB* cistrons (chromosomal origin) on pJC74 into the recombinant plasmid pANN202 carrying *hlyC* (plasmid origin) and then transforming into a nonhemolytic *E. coli* strain resulted in complementation and hemolytic colonies.

Further comparison of hly determinants of plasmid and chromosomal origin was made by Muller et al. (72). ^{32}P-labeled DNA probes prepared by nick translation of recombinant plasmids containing portions of *hlyA*, *hlyB*, and *hlyC* from pHly152 were used to detect homology between the pHly152 *hly* determinants and restriction digests of chromosomal DNA from various hemolytic strains (serogroups O4,

TABLE 2. Purification of *E. coli* AH

Method of purification	Growth medium	Final sp act (HU/mg)	Fold increase in sp act	% Recovery	LPS (μg/mg of protein/fold decrease)[a]	Carbohydrate (mg/mg of protein)
Ammonium sulfate and isoelectric point precipitation-Sephadex G-200 chromatography (79)	Nutrient broth	1,000,000	3,850	76	ND[b]	0
Ammonium sulfate and isoelectric point precipitation-Sephadex G-200 chromatography-ethanol precipitation (119)	Alkaline beef extract broth	109,226	874	26	ND	ND
Ultrafiltration-glycerol gradient ultracentrifugation (5)	CDM	230,000	20	111	1.0/2,176	12.1

[a] LPS was measured by the *Limulus* amoebocyte lystate test.
[b] ND, No data.

O6, O18, and O75). Hybridization patterns showed that the *hlyB* region was very conserved but that five different variations in *Eco*RI and *Hin*dIII restriction fragmentation patterns occurred within *hlyA*. Grouping of strains according to these digest patterns did not correlate with serogroup of the organism.

Welch et al. (117) cloned a 11.7-kilobase *Sal*I restriction fragment containing the hly determinant from the chromosome of the human *E. coli* strain J96 isolate into a vector plasmid. By restriction endonuclease mapping combined with mutagenesis by the Tn*1* transposon, the hemolysin determinant was localized to a 7.0-kilobase region. Hemolysin production was shown to be controlled by cistrons analogous to the hemolysin determinants in plasmids. The chromosomal *Eco*RI fragment containing *hlyA* hybridized to chromosomal but not to plasmid DNA of 17 other human isolates of hemolytic *E. coli* (serogroups O4, O6, O18, O25, and O75), thereby providing evidence that the hemolysin determinant in these strains is located on the chromosome. With restriction digest fragments encompassing the chromosomal hemolysin determinants as probes of colony blots, nonhemolytic strains failed to hybridize. This indicates that the hemolysin determinant was absent in these strains and that repression or mutation of the genes is not responsible for their nonhemolytic phenotype. Also, one β-hemolysin-producing strain (Hly^+in/Hly^-ex) did not hybridize, indicating that the genetic material encoding for its production is different from that encoding for AH.

Hacker et al. (43) isolated spontaneous type I (Hly^-in/Hly^-ex) and type II (Hly^+in/Hly^-ex) mutants of *E. coli* 536 (serogroup O6), an organism with chromosomal hemolysin determinants. Type I mutants were completely nonhemolytic and could not be complemented when transformed with *hlyA*, *hlyB*, or *hlyC* from pHly152. Type II mutants produced a very narrow zone of hemolysis on blood agar and were complemented to full hemolytic activity upon acquisition of *hlyB* by transformation, indicating that they were affected only in the *hlyB* cistron. Type II mutant DNA was shown by Southern hybridization to hybridize to restriction fragments containing *hlyA*, *hlyB*, and *hlyC* from pHly152, suggesting that at least part of each cistron was present. In contrast, DNA from type I mutants failed to hybridize to DNA from these cistrons, indicating that the type I mutants lack all of the hemolysin determinants. These investigators also detected sites distal to both ends of the chromosomal hemolysin determinant which hybridized with a 0.6- to 1.2-kilobase sequence distal to *hlyB* of pHly152. They speculated that the homologous distal regions common to hemolysin determinants of both plasmids and chromosome may be insertion sequences. Recombination between these sequences at opposite ends of the hly determinant followed by excision of the enclosed DNA was proposed as a mechanism for the production of type I mutants.

Even though both plasmid and chromosomal hemolysin determinants possess identical or similar DNA sequences, Welch et al. (115) showed differences in virulence of a strain of *E. coli* after receiving recombinant plasmids containing hemolysin determinant from several sources. Welch and Falkow (116) accounted for this difference by showing quantitative differences in hemolysin production. The region accounting for differences in hemolysin production and virulence was found in a 750-base pair *Ava*I fragment located in the region containing the *hlyC* cistron and where the probable transcription initiation area is located.

CHARACTERISTICS OF *E. COLI* AH

Numerous attempts have been made to purify *E. coli* AH. Although products of high activity have been obtained, purification has not been achieved; therefore, the products have been difficult to characterize.

Purification

Several groups have obtained partially purified *E. coli* AH from cells grown in either meat infusion broth (1, 53, 64, 79, 85, 118, 119, 121) or CDM (5). AH can be precipitated from culture filtrates with methanol (118), methanol and zinc (1), and ammonium sulfate (53, 64). Isoelectric precipitation has also been used by some (79, 119). Short and Kurtz (85) showed that AH can be separated from other culture components by gel filtration chromatography on Sephadex G-200 and Sepharose 6B. Hemolytic activity elutes from these columns in two peaks, indicating heterogeneity. They also reported that AH binds to DEAE-cellulose at pH 6.0 and can be eluted as a single peak by increasing ionic strength.

A summary of those attempts that present data indicating some purification is given in Table 2.

Rennie and Arbuthnott (79) obtained a highly active product with substantial purification. However, two components were detected by immunodiffusion, suggesting that some medium components were not removed. Characterization by polyacrylamide gel (3.7%) electrophoresis in sodium

dodecyl sulfate and urea could not be done because the hemolysin did not enter the gel. Electrofocusing was also attempted but the hemolytic fraction precipitated at its isoelectric point of pH 4.6 and could not be recovered without contamination by other nonhemolytic components.

Williams (119) used the procedure of Rennie and Arbuthnott (79) and obtained an approximately 250-fold purification and a specific activity of 4×10^4 hemolytic units (HU)/mg of protein. In agreement with others (64, 121), most of the AH activity was lost (>80%) during dialysis of the ammonium sulfate precipitate. By using a similar method but desalting by Sephadex G-25 gel filtration, Williams obtained an 874-fold purification and a specific activity of approximately 10^5 HU/mg of protein.

Cavalieri and Snyder (5) reported a simple two-step procedure for obtaining AH from CDM filtrates. The use of CDM has the advantage of eliminating medium proteins from the AH preparation. Their preparation was virtually free of lipopolysaccharide (LPS) contamination as measured by the *Limulus* amoebocyte lysate test. Electrophoresis of AH preparations in polyacrylamide gels under nondenaturing conditions was unsuccessful, probably due to the high molecular weight of the molecule. Electrophoresis after denaturing with sodium dodecyl sulfate and 2-mercaptoethanol and boiling revealed one well-defined protein band and another faint band. The major band was probably AH since it was absent after electrophoresis of similar preparations from a nonhemolytic mutant (S. J. Cavalieri and I. S. Snyder, unpublished data). Only one precipitin line was obtained by immunodiffusion of purified AH with an anti-hemolysin serum.

It is clear that hemolysin preparations of high activity can be obtained by classical methods of purification. However, except for one report (5) no attempt has been made to assess contamination with other materials such as endotoxin. Except for the study by Williams (119), good recovery of hemolytic activity was accomplished, indicating that the hemolysins prepared in nutrient broth and CDM tolerate the methods used for concentration and purification. More needs to be done on purification attempts, using newer technologies such as high-pressure liquid chromatography, and affinity chromatography with monoclonal antibodies should be evaluated.

Chemical Composition

The validity of the data on chemical composition is limited by the fact that studies have used partially purified preparations of AH. During purification hemolytic activity is always associated with major protein peaks as determined by absorbance at 280 nm or by chemical assay (Table 3). Rennie and Arbuthnott (79) reported that their preparation of AH was 95% protein by weight. Further evidence for the molecule being protein is that the hemolytic and cytotoxic activities of AH are destroyed by trypsin, pronase, and chymotrypsin (6, 79, 85, 95).

The presence of carbohydrate in preparations of AH may depend upon the medium in which it is produced. Carbohydrate is apparently absent from AH preparations prepared from complex media (79, 85, 121). However, Cavalieri and Snyder (5) demonstrated it in partially purified preparations from CDM. Whether this carbohydrate represents a contaminant or a component of AH is yet unclear. Carbohydrate is also found in AH from CDM after gel filtration through Sephacryl S-1000 followed by ion-exchange chromatography (G. A. Bohach and I. S. Snyder, unpublished data).

Treatment of AH with DNase, RNase, lecithinase, or lysozyme has no effect on AH activity, indicating that nucleotides, lecithin, or peptidoglycan do not comprise the active site (85, 95). Likewise, Rennie and Arbuthnott (79) noted the absence of organic phosphate in purified AH.

Lipids have not been detected in some AH preparations (5, 121). However, enzymatic treatment of AH with lipases destroys hemolytic activity (6), suggesting that a lipid component may be necessary for AH activity. Fatty acids, including β-hydroxymyristic acid, have been detected by mass spectrometry in an AH preparation prepared from CDM (Bohach and Snyder, unpublished data). This supports earlier work indicating the presence of endotoxin in partially purified AH preparations (5). It is unclear whether the presence of endotoxin represents contamination or whether the hemolysin is complexed with endotoxin.

TABLE 3. Chemical and physical characteristics of *E. coli* AH[a]

Study	Growth medium used	Chemical composition[b]				Mol wt	Heat stability[c]	
		Protein	Lipid	Carbohydrate	Nucleic acids		MIB	CDM
Jorgenson et al. (53)	AEB	P[d]	NT	NT	NT	$>1.5 \times 10^5$ 3×10^4–4×10^4	L	NT
Rennie and Arbuthnott (79)	NA	P[e]	UD	UD	UD	5×10^5–6×10^5	L	NT
Short and Kurtz (85)	AEB	P[d,f]	UD[f]	NT	UD[f]	$>3 \times 10^5$	NT	NT
Williams (119)	AEB	P[d]	NT	NT	NT	1.2×10^5	NT	NT
Zwadyk and Snyder (121)	MIB	P	UD	UD	NT	NT	L	NT
Cavalieri and Snyder (5)	CDM	P	UD	P	NT	$>3 \times 10^5$	NT	NT
Cavalieri and Snyder (6)	CDM	P[f]	P[f]	NT	UD[f]	NT	NT	L
Noegel et al. (76)	MIB	NT	NT	NT	NT	5.8×10^4	NT	NT
Lovell and Reese (64)	AEB	NT	NT	NT	NT	NT	L	NT
Smith (88)	AEB	NT	NT	NT	NT	NT	L	NT
Snyder and Koch (94)	MIB	NT	NT	NT	NT	NT	L	S

[a] Abbreviations: MIB, meat infusion broth; AEB, alkaline extract broth; P, present; UD, undetectable; NT, not tested; L, activity labile to heating 56°C for 1 h; S, activity stable to heating at 56°C for 1 h.
[b] Based on chemical analysis unless otherwise specified.
[c] 56°C.
[d] Based on absorbance at 280 nm.
[e] 95% protein by weight.
[f] Based on destruction or retention of activity after treatment with the appropriate hydrolytic enzyme (proteases, lipases, DNase, or RNase).

AH prepared in meat infusion media is insoluble at pH values of 4.0 to 5.0, indicating that AH is acidic with an isoelectric point of between pH 4.0 and 5.0 (85). In contrast, AH prepared from CDM does not precipitate when dialyzed in an acetate buffer, pH 4.0, but binds to DEAE-Sephadex, an anion-exchange resin, at pH 3.0 without substantial loss of activity (Bohach and Snyder, unpublished data). This indicates that AH produced in CDM is much more acidic than that from meat infusion media.

Physical Characteristics

Estimates of molecular weight of AH have ranged from $3–4 \times 10^4$ to $5–6 \times 10^5$ (Table 3). Williams (119) calculated a molecular weight of 120,000 by gel filtration and a value of 119,800 by sedimentation equilibrium with or without sodium dodecyl sulfate. Rennie and Arbuthnott estimated that nondenatured AH has a molecular weight of 5×10^5 to 6×10^5 by diffusion coefficient measurements and negative stain electron microscopy (79). AH from CDM is retained by a membrane filter with a 300,000-dalton cutoff, suggesting a value in excess of this figure (5). Recent data from our laboratory show that AH from CDM is voided by Sephacryl S-300 (exclusion limit, 1.5×10^6 daltons) but is retarded on Sephacryl S-1000. Noegel and co-workers (76), using a hemolysin plasmid and recombinants of it in minicells, showed that the processed protein encoded by the AH structural gene *hlyA* has a molecular weight of 58,000 under denaturing conditions.

The data indicate not only a wide variation in molecular weight of AH prepared in meat extract broth but a large difference between that prepared in meat extract broth and CDM. The >10-fold variation in the values reported for the molecular weight of AH suggests that in some preparations the molecule is multimeric or represents a complex with medium components or other bacterial components. The hemolysin has not been successfully analyzed or separated from other contaminating proteins by electrophoresis in the absence of denaturing compounds. Unfortunately these compounds inactivate AH. Therefore, the actual hemolytic protein has not yet been conclusively identified in culture supernatants. Immunochemical analysis with hemolysis-neutralizing monoclonal antibodies may solve this problem. Several groups (53, 85) have reported at least two molecular species based on elution profiles from gel filtration chromatography. The presence of carbohydrate and lipid in some purified AH preparations, and differences in isoelectric point, may be a reflection of different hemolysins or the influence of the medium in which AH is produced. Some workers (53, 97) have demonstrated the importance of a meat protein for hemolysin production and one group (53) indicated that bacterial protein constituted only a small part of the hemolytic preparation. Resolution of the molecular weight may require additional purification.

Requirement for Divalent Cations

Bamforth and Dudgeon (2) originally showed that the divalent cation calcium, strontium, or barium was required to demonstrate hemolytic activity in cultures of *E. coli*. Snyder and Zwadyk (95) showed that calcium or strontium but not barium or magnesium was required for hemolytic activity of AH. Others have confirmed the calcium requirement (80, 85). However, Lovell and Rees (64) and Smith (88) made no mention of a calcium requirement for activation of AH from alkaline extract broth. In fact, van den Bosch and co-workers (105) reported that addition of calcium did not affect hemolytic activity. One explanation for these differences is that alkaline meat extract broth medium contains small quantities of calcium (95) which may be sufficient to activate the hemolytic reaction and thus requires no exogenous addition.

The requirement for calcium in the hemolytic reaction is incompletely understood. Snyder and Zwadyk showed that calcium does not act by removing inhibitors of AH from the medium (95); therefore, the ion must activate AH or be directly involved in the hemolytic reaction. Calcium has been reported to change AH from elongated amorphous aggregates to regular spherical structures as observed by electron microscopy (80). Whether this change in shape results in activation is not known.

Short and Kurtz (85) found that addition of EDTA to the calcium-hemolysin-RBC reaction mixture during the incubation period immediately stops the hemolytic reaction and prevents additional hemolysis. Conversely, Rennie et al. (80) demonstrated that the hemolytic reaction is not inhibited if EDTA is added after the AH and calcium are allowed to react for at least 2 min before addition of RBC. These investigators (80) also showed that preincubation of hemolysin with calcium shortens the lag phase of the hemolytic reaction. These findings suggest that the activation of AH by calcium is not a rapid reaction and that calcium may be required for formation of a hemolysin-RBC complex or for maintaining this complex during the prelytic phase or for both. How this relates to the changes in shape of the hemolysin reported by Rennie et al. (80) is unclear.

Stability of AH

The effect of temperature on stability of AH is apparently dependent upon the medium in which it is produced, its purity, and its state of activation (Table 3). AH from meat infusion medium is inactivated by heating at 56°C for as little as 10 min (53, 64, 79, 88, 94, 121). However, Jorgensen et al. (53) showed that a small portion of AH resists heating at 56°C for 10 min. Elution profiles from Sepharose 6B suggest that the heat-resistant and heat-labile fractions may represent two different species of AH. AH produced in meat infusion is also labile at 37°C, although it takes up to 18 h for inactivation to occur (64, 88, 121). Smith (88) showed that pH affects temperature stability of AH. AH in meat extract medium incubated at 37°C is stable at pH 3.0 for 6 h but total inactivation occurs within 7 days. AH is less stable at pH 7.0 to 10.0 and is inactivated within 6 h. At pH 0.5, 1.0, and 5.0, the hemolysin is inactivated within 1 to 4 days.

The heat stability of AH produced in CDM is dependent upon the state of purification and the presence of calcium ions. Crude AH is stable at 56°C for 1 h in the absence of calcium ions (94) but is heat labile in the presence of 10 mM $CaCl_2$ (Cavalieri and Snyder, unpublished data). In contrast, partially purified AH is labile at 56°C in the presence or absence of $CaCl_2$ (6).

Although little work has been done on chemical inactivation, it is known that AH retains activity after treatment with 1% Formalin (88). In contrast, 3 to 6 M urea causes a 60 to 90% reduction in AH activity after overnight incubation (79).

Although it is possible that different hemolysins could account for differences in stability, it is more likely that the differences in heat stability represent the influence of other bacterial products or of medium components on the hemolysin. In support of this are the high-molecular-weight estimates for native AH, indicating that the molecule can readily form aggregates or complexes with bacterial or medium

components. Other data suggest that AH produced in meat infusion medium either acquires a protein component present in the medium or is a modified medium protein (53). Further, alkaline meat infusion contains small amounts of calcium, a possible explanation for some reports that indicate that the hemolysin from meat extract medium is active and heat labile in the absence of added calcium (79). In contrast, CDM lacks calcium. Activation by addition of calcium to AH in CDM changes AH from heat stable to heat labile. Our unpublished data (Bohach and Snyder) showing that β-hydroxymyristic acid and endotoxin are associated with hemolysin prepared from CDM suggest that AH may complex with other heat-stable bacterial components, such as LPS, and thereby confer heat stability to AH. Because calcium causes LPS to aggregate (77), displacement of LPS from the hemolysin molecule and reaggregation with other LPS molecules could result in a labile hemolysin. Purification of CDM AH and removal of a large amount of LPS (6) also result in a labile hemolysin (Table 3).

Kinetics of Hemolysis and Mechanisms of Action

The hemolytic reaction is characterized by three phases: (i) an initial lag phase, (ii) a phase of accelerated rate of hemolysis, and (iii) a phase in which the rate of hemolysis reaches a maximum, plateaus, and then decreases (80, 85, 121). AH is activated by calcium ions and the calcium concentration affects the length of the lag phase (80). Binding of hemolysin to the RBC occurs as evidenced by inhibition of lysis when hemolysin is preincubated with RBC ghosts (85). The rate of hemolysis is dependent upon AH concentration, temperature, pH, and the concentration of calcium and RBC (80, 85, 121). Maximum hemolysis and rate of hemolysis have been reported at temperatures ranging from 40 to 45°C, with reduced lysis at 20°C (85, 121). The optimum pH for AH activity has been reported to be 7.0 to 8.0 (85, 109, 121).

Several lines of data show that RBC lysis results from a single hit by AH (52, 85). When the RBC concentration is increased in the presence of a constant amount of AH, total hemolysis rises to a maximum and then remains constant. Furthermore, at high RBC concentrations, the absolute number of cells lysed is essentially independent of the total number of cells. In an excess of RBC, doubling of hemolysin concentration results in release of twice as much hemoglobin (52). Similar data were reported by Short and Kurtz (85).

Direct microscopic observation of the hemolytic reaction provides some insight into the lytic process but has not offered any clues as to mechanism of lysis. Phase-contrast microscopy shows that the RBC undergoes spherocyte formation shortly after AH is added (85). As observed by scanning electron microscopy, multiple small projections occur on the membrane surface concurrently with spherocyte formation. These projections probably do not represent multiple hemolysin hits on the RBC since at very low AH/RBC ratios a significant population of RBC still show these multiple projections (52). Jorgensen et al. (52) suggested that a single AH molecule bound to an RBC might act as an ionophore and create a membrane channel. The resulting influx of ions could trigger reactions inside the cell. They proposed that formation of multiple projections might occur through activation of ATPase by an influx of calcium, thereby affecting the spectrin-actin cytoskeleton, or by activation of a lipase which would affect the lipid components of the membrane. However, there are no data to support or refute their proposal. Another possibility is that AH could enter the cell and trigger an internal reaction. The ability of AH to enter the RBC and directly alter an internal reaction must be questioned because of its high molecular weight. Like other toxins, AH may bind to the membrane, allowing a subunit to enter the RBC, but there is no evidence that the AH molecule contains dissociable binding and active subunits. The reducing agents mercaptoethanol, ascorbic acid, and dithiothreitol do not inhibit hemolysis, suggesting that oxidation of a cellular component is not involved in the lytic reaction (85). Rennie et al. (80) showed that the lag phase for hemolysin was inversely proportional to the logarithm of hemolysin concentration, indicating a nonreversible nonenzymatic mode of action. Others (52) have suggested that AH has a saponin-like effect.

The site of action on the RBC is unknown. Smith (88) reported that ox, sheep, horse, pig, rabbit, guinea pig, chicken, and human cells are equally susceptible to the β-hemolysin and AH. However, Rennie and Arbuthnott (79) found differences in sensitivities of RBC of several mammalian species to AH. Sheep and rabbit RBC are most susceptible, human and mouse RBC are less susceptible, and fish RBC are virtually resistant to the hemolytic action of AH. The broad susceptibility pattern of RBC from different species suggests that AH reacts with residues common on many types of RBC membranes. Short and Kurtz (85) reported that neither cholesterol nor lecithin inhibits the action of AH on RBC, suggesting that neither is involved in hemolysis or serves as a receptor site.

BIOLOGICAL EFFECTS OF AH

Hemolysin Production and Extraintestinal Infections of Humans

Several studies have shown that hemolytic *E. coli* cells are more frequently isolated from extraintestinal infections (UTI, bacteremia, peritonitis, appendicitis) than from the feces of healthy individuals (Table 4). In the only prospective study, Cooke and Ewins (9) reported that 47% of *E. coli* isolated from introital swabs (from the external urethral meatus) of patients who later developed UTI were hemolytic whereas only 7% of the *E. coli* cultures obtained from women who did not develop UTI were hemolytic. This suggests that colonization before development of infection may be enhanced by hemolysin production. The association of hemolytic *E. coli* with extraintestinal infections led several investigators to ask whether hemolytic *E. coli* isolates are more virulent than nonhemolytic isolates and whether the virulence factors of the hemolytic strain are different from those of virulent nonhemolytic strains.

Hemolysin-producing *E. coli* isolates are found primarily but not exclusively in serogroups O4, O6, O18, and O75 (9, 26, 48, 86, 102). Hughes et al. (48) reported that serum resistance among serogroups O6, O18, and O75 is strongly associated with hemolysin production. The association between serotype and hemolysin production in fecal isolates of *E. coli* was examined by DeBoy et al. (13), who showed that most of the serotype O6 isolates (85%) produced AH. Several groups have shown that hemolytic *E. coli* cells often hemagglutinate RBC (25, 35, 46, 63, 69) and possess fimbriae (4) and K antigen (25, 42, 46). Hughes et al. (46) reported that *E. coli* strains of high potential virulence for the urinary tract generally are hemolytic, serum resistant, and toxic for mice, adhere to urinary tract cells, and cause mannose-resistant hemagglutination.

van den Bosch and co-workers (107) compared the relative importance of serotype, hemolysin production, hemag-

glutination patterns, and anatomical origin of human *E. coli* isolates with virulence in a mouse model (103). Serotypes O2, O6, and O18ac strains were commonly associated with virulence, whereas strains of serotype O75 were rarely virulent. The presence of K or H antigens did not seem to be associated with virulence. However, virulent strains were more often hemolytic or hemagglutinating than were avirulent strains. van den Bosch and co-workers showed that hemolysin production is an important virulence factor in strains which are nephropathogenic for mice, whereas hemagglutination is an important factor for strains with generalized virulence. Further evidence for the importance of hemolytic *E. coli* in UTI is that the frequency of isolation of hemolytic strains increases in more critical clinical situations: i.e., normal feces < asymptomatic bacteriuria < cystitis < pyelonephritis.

In an attempt to assess the importance of hemolytic *E. coli*, several groups showed an association between hemolysin production and dermonecrotoxicity in mice (86) and rabbits (9), cytotoxicity for chicken embryo fibroblasts and human fetal fibroblasts (8, 9), virulence for embryonated eggs (48, 68, 69, 104), toxicity for mice (42, 44, 46, 47, 88), and virulence for mice (88, 103, 104).

These studies show that hemolytic *E. coli* isolates are a common source of extraintestinal infection even though they are found in a small percentage of fecal cultures. Further, hemolysin production alone does not always equate with virulence, but it may be a decisive factor in virulence of many nephropathogenic strains.

Immunology of AH

Several groups of workers have shown that AH-neutralizing antibodies are produced in animals (5, 22, 54, 58, 63, 88). Smith (88) showed that antibody to AH neutralizes the hemolytic activity of strains that produce AH but not the hemolytic activity of strains producing only cell-bound (β) hemolysin. In contrast Rennie and Arbuthnott (79) reported that antiserum to AH prevented hemolysis by a strain which produced ony cell-bound hemolysin, suggesting that in some cases AH may be a cell-free form of β-hemolysin.

TABLE 4. Association of hemolytic *E. coli* with disease

Study	% Hemolytic *E. coli* isolated from:			
	Healthy humans[a]	Patients with:		
		UTI[b]	Septicemia[c]	Other EI infections[d]
Cooke and Ewins (9)	14	44	ND[e]	ND
Green and Thomas (35)	6	38	ND	ND
DeBoy et al. (12)	7	35	50	35
Minshew et al. (68)	5	49	35	56
Evans et al. (26)	ND	42	29	ND
Brooks et al. (4)	9[f]	43	ND	ND
Dudgeon et al. (20)	13	45	ND	ND
Hughes et al. (47)	5–10	35	ND	ND
Hacker et al. (44)	12	33	ND	ND
Nimmich et al. (74)	ND	36[g]	ND	ND
Vahlne (102)	9	26	ND	10–19

[a] Fecal isolates unless otherwise specified.
[b] Urine isolates unless otherwise specified.
[c] Blood isolates.
[d] Miscellaneous extraintestinal infections (EI) including wound, sputum, drainage, or abscess isolates.
[e] ND, No data.
[f] Periurethral isolates.
[g] All rough strains.

Smith (88) reported that antibodies to AH are present in healthy adult animals of several species and can be passively transferred in colostrum. Antibodies which neutralize AH are also found in healthy humans and in patients with hemolytic *E. coli* infections (22, 58, 88). Individuals in the latter group have higher neutralizing antibody titers than healthy persons (22, 58). The average antibody titer in infected persons rises in proportion to the severity of the infection: i.e., pyelonephritis > cystitis > asymptomatic bacteriuria (22). These data make it clear that AH is antigenic and that antibody is produced during infection.

Immunization of mice with hemolytically active AH protects against lethal challenge with AH from homologous or heterologous strains (58, 88). Passive immunization against AH also protects mice against lethal infection with hemolytic *E. coli* cells (63). Antibody to AH also protects mice from lung edema caused by hemolytic *E. coli* (24). However, immunization with heat-inactivated hemolysin does not protect animals against challenge with hemolytic *E. coli* or AH (58). The loss of antigenicity by a hemolytically inactive preparation and the ability of antisera containing neutralizing antibody to protect against toxicity suggest that the hemolytic site of AH is also responsible for toxicity.

Much needs to be done to gain a better understanding of the antigenicity and immunogenicity of the *E. coli* hemolysin(s). It is clear that AH is antigenic and that polyclonal antibody to AH prevents some of the pathology associated with the hemolysin and against lethality of hemolytic *E. coli* infections. Also, antibody is produced during infections and titers correlate with severity of infection. Although this suggests a role for AH as a virulence factor for *E. coli*, interpretation of these results must be made with caution because the AH preparations used were not purified. Monoclonal antibody to AH (Bohach and Snyder, unpublished data) does prevent hemolysis and cytotoxicity for human peripheral leukocytes.

Effect of AH on Animals and Cells

Investigations into the effect of AH on animals and cells are largely inconclusive because of the use of crude culture supernatants that were poorly characterized with respect to purity and hemolytic titer. In addition, assays for the presence or absence of other toxins such as endotoxins, enterotoxins, and other cytotoxins were not done. However, most of the studies suggest that AH is toxic to cells.

Both Smith (88) and Khan et al. (58) showed that crude culture supernatants that contain AH are toxic and lethal for mice. Intravenous injection of hemolytic culture supernatant into mice results in lethargy, labored respirations, and hemoglobinuria. Death often occurs within 6 h after injection. Necroscopy of these animals revealed lung edema and bladder hemoglobinuria as the prominent pathological features. These effects are prevented by passive immunization with antibody to crude AH or by active immunization with sublethal amounts of crude AH. Both the lethal factor and the hemolytic activity in culture filtrates were neutralized by heating (56°C for 10 min). Supernatants from nonhemolytic strains of *E. coli* and strains that produce only cell-bound (β) hemolysin are not lethal when injected into mice. Neutralization by antiserum, heat lability, and lack of activity by nonhemolytic cultures suggest that AH might have caused

the pathology. The dose of AH used in these experiments is not clear but it appears that the minimum lethal dose used by Smith (88) was approximately 20 to 30 HU. Rabbits and guinea pigs challenged with AH (300 to 1,000 HU) are affected in a manner similar to that described above for mice (88). Dermonecrosis with hard palpable swelling results from intradermal injection (88).

These findings (58, 88) contrast those of Rennie and Arbuthnott (79), who found that intravenous injection of up to 500,000 HU of purified AH was nontoxic for both mice and rabbits. They speculated that the difference may be due to the relative states of purity of the AH preparations.

Chaturvedi and co-workers (8) reported that monkey kidney and mouse embryo cells are unaffected by culture filtrates from a strain of hemolytic *E. coli* whereas chicken embryo fibroblasts are highly susceptible. However, the cytotoxic activity was not destroyed by heating for 1 h at 56°C, indicating that the effect was probably not due to AH. These authors further fractionated AH preparations, using calcium and alumina gels, and point out that the fraction which did not bind (nonhemolytic) to the gel was cytotoxic. Preparations from nonhemolytic *E. coli* did not show cytotoxic activity. This work points out that other cytotoxic factors may be present in hemolysin preparations.

Emody et al. (25) reported that an ammonium sulfate-precipitable fraction of a hemolytic *E. coli* supernatant was cytotoxic for rabbit granulocytes. However, the effect was seen primarily after 60 min, which is quite different from the effect described by others for AH (6, 31). Emody did not report the hemolytic titer of his preparation or the purity.

More recently, Cavalieri and Snyder (5, 6) showed that AH purified from CDM by a glycerol gradient is cytotoxic for mouse fibroblast (3T3) cells and for human peripheral leukocytes. As little as 5 HU of AH (23 ng of protein) kills 10^5 to 10^6 cells. Cytotoxicity was measured by trypan blue uptake and by lysis. Cytotoxicity was demonstrated after as little as 5 min of exposure, followed single-hit kinetics, and was neutralized by pretreatment of AH with antiserum. This effect was not the result of endotoxin contamination. Treatment of AH with proteases and lipase inactivates both the hemolytic and leukotoxic effects of AH (6). Both hemolytic and leukotoxic activities are inactivated at the same rate by heating at 56°C. In addition, the hemolytic and leukotoxic activities copurify. In contrast to the study by Chaturvedi et al. (8), these data suggest that hemolysis and cytotoxicity are caused by the same or a similar factor. Subsequent work by Bohach and Snyder (unpublished data) confirms that AH is cytotoxic for human peripheral leukocytes. A monoclonal antibody preparation that neutralizes hemolysis also prevents cytotoxicity.

Cavalieri and Snyder (7) also showed that AH at concentrations below that which cause trypan blue uptake affects leukocyte functions in vitro. Pretreatment of neutrophil-enriched human leukocyte preparations with sublethal doses of AH decreases their ability to phagocytose opsonized *Staphylococcus epidermidis* and LPS particles by 30 to 70%. Chemotaxis of leukocytes is likewise decreased by 60%. AH activates neutrophil oxidative metabolism, resulting in an intense burst of chemiluminescence (7). The effect is time and dose dependent and precedes death of the leukocyte (6).

The mechanism by which AH kills granulocytes and other cells and modifies the functions of polymorphonuclear leukocytes is not known. It seems likely, however, that the initial interaction causes damage or stimulation of the cell membrane. Damage is clearly evidenced by cytotoxicity (trypan blue uptake) and lysis. At low doses, membranes are modified, resulting in decreased adherence and chemotaxis. This view is also supported by the finding that exposure of phagocytes to AH activates oxidative metabolism, resulting in an intense burst of chemiluminescence (7).

Gadeberg and co-workers (31) compared the cytotoxic effect of culture supernatants containing AH with washed intact hemolytic cells from the same *E. coli* culture. Hemolytic *E. coli* cells are toxic for leukocytes, with monocytes being the most sensitive. Granulocytes are also sensitive but lymphocytes are relatively resistant. This cell-associated toxic effect is dose and time dependent and heat sensitive and occurs in the presence of autologous plasma. Culture supernatants from this AH-producing *E. coli* strain cause similar cytotoxicity except that lymphocytes appear to be more sensitive to the AH preparation than to the bacterial cells. The AH-induced effect is neutralized by autologous plasma, and Gadeberg and co-workers suggest that AH may be less likely to affect phagocytic cells in vivo than the cell-associated factor. Gadeberg et al. (31) did not determine whether the cell-associated and AH-induced toxic effects are caused by the same hemolysin. The cell-associated factor could be the distinct β-hemolysin of *E. coli* (88) or it may represent a cell-bound form of AH that is only partially exposed to neutralizing factors in plasma. However, data obtained with mutants in *hlyB* indicate that mutants in $hlyB_b$ are phenotypically identical to β-hemolysin-producing *E. coli*. Since the toxic effects were different for lymphocytes they might be distinct cytotoxins.

In a subsequent report, Gadeberg and Orskov (30) reported that 59 of 117 strains of hemolytic *E. coli* possess cell-associated cytotoxicity for human granulocytes. However, some nonhemolytic mutants derived from hemolytic strains lost cytotoxicity, whereas other mutant strains lost cytotoxic activity without losing hemolysin production. Although cell-associated cytotoxicity was not observed with any of 62 nonhemolytic strains tested, supernatants from some nonhemolytic strains were cytotoxic. All strains which produce AH (cell-free hemolysin) were highly cytotoxic for human leukocytes regardless of the toxic capacity of the bacterial cells. This provides further evidence that AH is cytotoxic but also demonstrates that multiple toxins can be synthesized by a single strain of *E. coli* and can complicate studies on AH cytotoxicity. Whether the cell-associated cytotoxicity is due to AH is still to be determined.

Virulence of Hemolytic *E. coli* in Animal Models

Edema disease of swine has been associated with the presence of AH-producing *E. coli* in the gut (36). This severe disease is characterized by widespread edema, neurological abnormalities, generalized angiopathy, and a high fatality rate. This generalized symptomatology is typical of infectious diseases in which toxemia is generated by the causative agent. Kurtz and Short (59) studied the pathogenesis of edema disease and its relation to hemolytic *E. coli*. They compared the effects of intravenous injections of AH from crude *E. coli* culture supernatants, autolysates of cells from the same *E. coli* culture, and endotoxin extracted from a different *E. coli* strain. They found that most of the pathology in the acute phase of edema disease of pigs is due to LPS. The vascular lesions and related symptoms of the chronic phase are caused by a factor present in crude AH preparations and cell autolysates. AH is probably not this factor because it should not be present in autolysate preparations. However, Kurtz and Short do not believe that the association between hemolysin production and pathogenic-

ity is fortuitous. Though there are no supporting data, they speculate that AH may enhance adherence of *E. coli* to the intestinal mucosa or damage cells in the intestine, thereby providing a portal for endotoxin and an additional vasculature-damaging toxin to enter the circulation. However, Shibl and Gemmell (84) were unable to show an association between hemolysin production and adherence to uroepithelial cells. Cavalieri and Snyder (7) showed that AH treatment of leukocytes decreases binding of opsonized bacteria.

Smith and Linggood (93) reported that AH production does not contribute to the virulence of enteropathogenic *E. coli* from pigs whereas enterotoxin production and K88 antigen production are required for diarrhea. Hemolytic strains cured of their Hly plasmid are as virulent as the wild-type strain when introduced orally. Both hemolytic and nonhemolytic organisms produce diarrhea and edema disease of equal severity. This is supported by reports that fluid accumulation is not observed when AH or lung toxin (see below) is injected into rabbit ligated intestinal segments (57, 79).

Emody et al. (25) could not demonstrate substantial differences in toxicity between AH-producing cultures and their nonhemolytic derivatives by intravenous injection of mice. However, when introduced intraperitoneally or intranasally, hemolytic organisms were clearly more toxic (25, 57). A hemorrhagic lung edema and lethal effect similar to that observed by Smith (88) was reported. The factor toxic for mouse lungs was partially characterized by Ketyi et al. (57), who found it to be cell bound and heat labile. The toxin could be extracted in low yields from *E. coli* cells and possessed cytotoxic activity for various tissue culture cell lines. The cytotoxin has a molecular weight of 100,000 but, due to the low sensitivity of the mouse lung toxicity assay, they could not confirm that the lung toxin and cytotoxin were identical. Although the lung toxin was not found in culture supernatants, several lines of evidence suggest that it may be a cell-bound form of AH. First, all hemolytic strains but none of the nonhemolytic strains of *E. coli* cause lung toxicity (57). Second, antisera prepared against either crude AH or purified cytotoxin are cross-neutralizing (24). In addition, wild-type AH-producing *E. coli* strains that are positive in the mouse lung assay lose this activity when cured of their Hly plasmid. Conversely, transconjugants that received the Hly plasmid in mating experiments acquire AH and lung toxicity simultaneously.

Fried and Wong (28) reported that 27 to 54% of rats intravenously injected with hemolytic *E. coli* develop pyelonephritis and bacteriuria. The kidneys of these rats showed multiple abscesses as well as tubular and interstitial infiltration by polymorphonuclear leukocytes and lymphocytes. In contrast, rats injected with nonhemolytic *E. coli* did not develop pyelonephritis or bacteriuria and their kidneys appeared normal. However, the cultures used were of different serogroups and isogenic nonhemolytic strains were not studied. In a subsequent study, Fried et al. (27) demonstrated that approximately 40% of rats and mice intravenously injected with hemolytic *E. coli* developed pyelonephritis. None of the rats and only 7% of the mice developed pyelonephritis when injected with a nonhemolytic mutant of the same strain of *E. coli*.

In a later study, Fry and associates (29) described the ultrastructural changes in kidneys of rats challenged intravenously with hemolytic *E. coli*. Four hours after injection, a few proximal tubular cells contain swollen mitochondria. After 6 h, dilation of endoplasmic reticulum and nuclear membranes in proximal tubular cells resulted in the formation of clear intracellular vacuoles. After 12 h generalized mitochondrial swelling and a decrease in the number of lysosomes were observed. Lysosomes were apparently in the process of lysing degenerating mitochondrial structures. Over the next 36 h progressive cellular damage, nuclear irregularity, and loss of nuclear membrane integrity occurred. All of the pathology occurred in the absence of observable intrarenal bacteria, suggesting that the degenerative changes were due to a bacterial toxin. However, these investigators did not use a nonhemolytic mutant as a control to determine the role of hemolysin in causing those effects nor have they demonstrated the tissue pathology with AH. The data reported do correlate with the in vitro effects of hemolytic *E. coli* on lysosomes as reported by De Pauw et al. (17). These workers (17) showed that incubation of hemolytic *E. coli* with lysosomes from rat renal cells in vitro results in disruption of lysosomes and release of lysosome-associated enzymes. They hypothesized that the ability of hemolytic *E. coli* to alter lysosomal membranes could account for their increased virulence in renal infections. Whether this effect is due to hemolysin is unclear, in part because the nonhemolytic *E. coli* isolates used as controls were not derivatives of the hemolytic strain. Further, washed *E. coli* cells and not AH were used. Though AH or a cell-bound form of hemolysin could have been produced during the 3 h incubation, no attempt to measure hemolytic activity was reported.

van den Bosch et al. (103) showed differences in nephropathogenicity of *E. coli* urinary tract isolates by quantitating the organisms in various tissues of mice after intravenous challenge. In this model human isolates of *E. coli* fall into three main groups on the basis of relative virulence. With group I organisms, viable counts in the mouse kidney fall rapidly after injection and remain low during the 8-h experimental period. These strains are avirulent and rarely kill mice even after intravenous challenge of more than 10^9 cells. With group II organisms, the viable count in the kidney also falls initially but begins to increase after 4 h and reaches or surpasses the level of the initial inoculum by 8 h. The 50% lethal dose of these strains is approximately 10^8 organisms; most mice die within 4 days. Viable counts of group III *E. coli* in the kidney rise rapidly and remain high. This group is the most virulent, with a 50% lethal dose of approximately 10^7 cells. Most mice injected with group III organisms die within 8 h. Viable counts of organisms in groups I and II in other tissues (blood, spleen, and liver) yield low counts. However, large numbers of group III organisms are present in these organs. In this model strains of *E. coli* in group II are specifically virulent for mouse renal tissues (nephropathogenic), whereas group III strains show a more general virulence.

van den Bosch and associates (106) compared AH-producing and nonhemolytic *E. coli* isolated from the urinary tract of humans in the mouse model described above. They found that 20 of 24 AH-producing strains were in virulence groups II and III, whereas the majority of 31 nonhemolytic strains were in the avirulent group I. However, 11 nonhemolytic strains were in groups II and III. Within each group, the hemolytic strains killed mice more rapidly than nonhemolytic strains did. Hemolytic strains of group II that were mutagenized or cured of their Hly plasmid, thereby rendering them nonhemolytic or less hemolytic without changing any other characteristic, behaved as group I strains. In contrast, reduction or abolition of AH-producing ability only slightly increased the mouse survival time of group III strains but did not change their group III status. However,

group III strains which had reduced AH-producing capacity and also lost their K antigen behaved as group I strains. Hemoglobinuria which is characteristically produced by injection of AH or AH-producing strains was not produced by the nonhemolytic group III strains. These workers concluded that AH production is a decisive virulence factor in group II *E. coli* strains but not in group III strains.

To further test the importance of hemolysin production in the nephropathogenic group II strains, Waalwijk et al. (111) treated a hemolytic group II strain, designated P673, with actinomycin D to eliminate the Hly plasmid. The nonhemolytic mutant (P673/II) lost virulence and was similar to group I (avirulent) strains in the mouse model. A hemolytic *E. coli* transconjugant produced by mating P673 with an avirulent K-12 strain was not virulent. However, reintroduction of the 41-Mdal Hly plasmid into p673/II resulted in acquisition of nephropathogenicity, emphasizing the multifactorial nature of *E. coli* virulence. Waalwijk and de Graaff (108) later showed that Tn*5* transposon insertions that reduce or eliminate AH production result in reduction or loss of nephropathogenicity. Insertion of transposons without affecting hemolysin production did not alter virulence. This provided more direct evidence that AH and not some other function of the Hly plasmid was responsible for virulence.

Emody and co-workers (25) reported similar data, using Hly^+ wild-type strains, Hly^- derivatives, and Hly^+ transconjugants. These investigators also showed that Hly^- strains are cleared more efficiently from the blood of mice injected intravenously than Hly^+ strains are (10 to 10^3-fold difference).

In other experiments Waalwijk et al. (110) simultaneously injected a group II hemolytic strain of *E. coli* and a group I Tn*5*-induced nonhemolytic mutant strain into mice. Both strains (hemolytic and nonhemolytic) increased in number. The kinetics of total kidney bacterial count, including that of the group I nonhemolytic strain, resembled that of group II nephropathogenic strains, suggesting that AH produced by the hemolytic *E. coli* strain acts as a helper for the nonhemolytic mutant in vivo. AH and $FeSO_4$ substituted for viable hemolytic bacterial cells in providing this helper function for the group I nonhemolytic strain, indicating that lysis of RBC and release of iron might be responsible. Iron salts enhanced the growth of *E. coli* in vitro, further suggesting that the helper function of AH may be one of obtaining iron for growth through RBC lysis.

Linggood and Ingram (63) obtained similar results, using the 50% lethal dose for mice as an indicator of virulence for *E. coli* administered by the intraperitoneal route. Acquisition of an Hly plasmid by a nonhemolytic strain resulted in a 10-fold increase in virulence. Similar to the data of Waalwijk et al. (110), they showed that Hly^- strains of *E. coli* proliferate as well as Hly^+ strains when simultaneously injected into mice. Further, mice injected with AH are more susceptible to Hly^- strains. Whereas heat-inactivated AH or a similar preparation from an Hly^- strain did not act as a helper for nonhemolytic avirulent strains, phenylhydrazine hydrochloride (which causes hemolytic anemia but not death in mice), iron, manganese salts, or hemoglobin do serve as helpers. As further evidence that AH was contributing to enhanced growth of *E. coli*, they showed that immunization with AH protected mice against the increased lethality associated with hemolytic *E. coli*. Protection afforded by immunization with AH was reversed if iron was injected before challenge with Hly^+ *E. coli*. These data support the concept that AH is a virulence factor and that it may enhance the virulence of *E. coli* for mice by enabling them to obtain growth factors such as iron through the lysis of RBC. This would result in rapid bacterial proliferation and ultimately death of the animal.

Recently, Welch and associates (115) demonstrated that acquisition of the DNA sequence encoding for AH by an avirulent, nonhemolytic, fecal strain of *E. coli* converts it to a virulent strain as measured by the rat peritonitis model of Weinstein et al. (114). The 11.7-kilobase *Sal*I restriction endonuclease fragment containing the *hly* genes was obtained from the chromosome of a virulent hemolytic strain (J96) of *E. coli* isolated from a human patient with a UTI. The fragment was introduced into a vector plasmid and the resulting hybrid plasmid was used to transform an avirulent, nonhemolytic, wild-type fecal strain of *E. coli* (J198). The hemolytic transformant (WAF107), injected intraperitoneally into rats, showed a marked increase in virulence and mortality over J198. The virulence of WAF107 was comparable to that of strain J96. Site-specific mutagenesis of the *hlyA* cistron in WAF107 by the *Tn1* transposon resulted in a nonhemolytic and avirulent transformant. Because the time course of infection caused by the virulent transformant WAF107 was somewhat longer than that of the virulent J96, these authors were unable to conclude that the acquisition of *hly* genes itself could convert avirulent fecal strains to virulent strains typical of those found in extraintestinal infections. Introduction of the *hly* genes from an Hly plasmid (pHly152) of a strain of *E. coli* isolated from mouse feces into the avirulent J198 strain did not result in increased virulence. This suggested that similar hemolytic phenotypes resulting from close genetic homology among AH-encoding regions of different *E. coli* strains may not ensure that both have equal pathogenicity.

The observations of Welch et al. (115) and Hull et al. (49) suggest that factors in addition to hemolysin are necessary for virulence in *E. coli*. The latter group showed that the virulence of hemolytic *E. coli* J96 for chicken embryos was lost by nonhemolytic mutants but was restored in Hly^+ recombinants. However, transfer of the hemolysin determinant to an avirulent *E. coli* K-12 strain did not confer virulence.

The work of Emody et al. (23) also suggests a role for *E. coli* AH in virulence. These workers obtained in vivo transfer of the Hly plasmid from *E. coli* into *P. morganii* in mice by oral administration of both organisms. The hemolytic *P. morganii* transconjugants isolated from the intestines of the mice were more virulent in the mouse lung toxicity test and for chicken embryos than either the *E. coli* K-12 hemolytic donor or the original nonhemolytic *Proteus* recipient.

Hacker et al. (42) added significantly to studies on the role of AH in toxicity of *E. coli*. Large doses of *E. coli* isolated from the human urinary tract were injected intraperitoneally into mice and deaths were recorded 6 to 20 h after inoculation. This time period was chosen so that deaths resulting from toxicity (<24 h) could be distinguished from deaths due to active infection (>24 h). Of the 69 isolates studied, Hly^+ strains were significantly more toxic than Hly^- strains. A spontaneous mutant with deletions in the chromosomal region containing genes for hemolysin production and for mannose-resistant hemagglutination (mrh^-) was much less toxic than the parent strain. The O and K antigens of the mutant were identical to those of the wild type. Similar virulence and avirulence were observed for the wild type (hly^+/mrh^+) and mutant (hly^-/mrh^-), respectively, in endotoxin-resistant mice, indicating that a change in endotoxin was not responsible for the change in virulence. Introduction

of cloned chromosomal hly determinants from three serogroups (O6, O18ac, and O75) into the nontoxic *hly*$^-$/*mrh*$^-$ mutant resulted in acquisition of different degrees of toxicity depending upon the source of hly determinant. Introduction of cloned hly determinants of plasmid origin into this mutant only slightly increased toxicity. Chromosomal hly determinants were more efficient at complementing hemolysin production but, depending upon the serogroup of the donor strain, were variable in the degree of complementation (O75 > O18ac > O6). This phenomenon was not the result of quantitative differences in hemolysin production. Hacker et al. (43) showed that mutants with deletions in *hly* that caused loss of Hly_{in} and Hly_{ex} also lost Mrh. Mutants with smaller deletion affecting only Hly_{ex} were Mrh$^+$. This suggested a close linkage between hly and mrh determinants on the chromosome. Low et al. (65) confirmed the close linkage between *hly* and *mrh* in several serotype O4 and O6 urinary tract isolates. Most fecal *E. coli* did not contain these determinants. This work is significant because it stresses the importance of multiple factors in virulence of *E. coli*.

Berger et al. (3) as well as Muller et al. (72) reported that the structural gene (*hlyA*) for hemolysin from different isolates of hemolytic *E. coli* shows some variation in nucleotide sequence. This suggests that differences in virulence of *E. coli* isolates or in toxicity of the hemolysin molecule might be a reflection of amino acid composition. All of the evidence accumulated with Hly$^+$ and Hly$^-$ mutants support the concept that hemolysin production is an important virulence attribute of *E. coli* but it may not be the sole attribute. Whether there are different toxicities associated with different hemolysin preparations is unknown or whether quantitative differences in hemolysin production can account for the differences is still unclear. As described earlier, Welch and Falkow (116) have identified a region in the hemolysin chromosomal determinant which accounts for quantitative differences in hemolysin production and virulence.

The effects of hemolytic *E. coli* on experimental animal models have been studied extensively in an attempt to show the relevance of hemolysin production. The data show that hemolysin production by enteropathogenic porcine strains is not important in edema disease and diarrhea; however, in experimental pyelonephritis and peritonitis hemolysin production or a factor associated with the hemolysin undoubtedly contributes to severity of the disease, albeit by an as yet unknown mechanism. The specific role that AH plays in the virulence of *E. coli* is not clear.

SUMMARY AND CONCLUSIONS

The association of hemolytic *E. coli* with extraintestinal disease of humans has been documented by several investigations. In addition to production of hemolysins, these pathogenic isolates are likely to possess other potential virulence factors, e.g., serum resistance, adherence (hemagglutination) factors, and certain O antigens. This results in the conclusion that virulence of *E. coli* for humans is multifactorial. In fact, there is now evidence that virulence factors in some strains may be linked. This may be true for the genes encoding for hemolysin production (*hly*) and for those encoding for mannose-resistant hemagglutination (*mrh*). Some data suggest that for nephropathogenic organisms hemolysin may be a decisive factor in virulence. Direct evidence that hemolysin plays a role in production of disease comes from studies showing that virulence of hemolytic strains is lost by nonhemolytic mutants of the same strain. In addition, culture supernatants from AH-producing strains and partially purified preparations of AH are cytotoxic for leukocytes, chicken embryo fibroblasts, and mouse fibroblasts. Further, hemolytic strains are more toxic to mice than are nonhemolytic *E. coli* strains. Given the present state of knowledge, the hemolysin could contribute to the disease process in at least three ways. First, since AH is cytotoxic for tissue cells in vitro, it may damage cells in vivo and contribute directly to tissue pathology. Second, because it affects leukocytes and their function, the hemolysin may allow or enhance survival of the organism by affecting host defense mechanisms. Third, lysis of erythrocytes by AH may be a mechanism by which the organism obtains iron for enhanced growth in vivo and perhaps for continued synthesis of hemolysin. Further experiments are needed to clarify these proposed roles and to investigate other potential roles.

We do not know the amount of hemolysin in tissue nor do we know if it is active in tissue. It is apparently produced in vivo since patients with *E. coli* infections show a rise in anti-AH-neutralizing antibody titer. Studies of AH effects in vitro are still somewhat suspect because pure hemolysin has not been isolated in an active form and some of the data suggest that other cytotoxins may be present. The question as to whether other cytotoxic factors may contribute to some of the noted effects remains unanswered.

Much information on the production and characteristics of AH has been obtained. Hemolysin is produced during the logarithmic phase of growth in meat infusion, casein hydrolysate, and CDM. Some evidence indicates that a protein component of meat extract is required for production of AH or for release of hemolysin from the bacterial cells. However, no such factor is required for production of AH in CDM. AH production has been at least partially defined by the use of genetic tools. AH production results from hly determinants on plasmids, as in *E. coli* strains isolated from animals, or from hly determinants on the chromosome, as commonly occurs with human isolates. No less than three cistrons are required for production and transport of the hemolysin from the cell. One cistron (*hlyA*) codes for production of the hemolysin precursor. A second (*hlyC*) codes for activation and transport through the cytoplasmic membrane to the periplasm. The third cistron, *hlyB*, is responsible for transport of the hemolysin from the periplasm to the exterior of the cell. Recently, the functions of *hlyB* have been further separated in that $hlyB_a$ codes for a protein that translocates the hemolysin through the outer membrane whereas $hlyB_b$ codes for a protein responsible for release of the hemolysin from the outer membrane to the external medium. How these proteins function is not clear. Studies show that mutations in the $hlyB_b$ cistron result in a phenotype possessing only cell-bound hemolysin. This is presumably the result of a defective system for releasing AH from the outer membrane.

The *hlyA* cistron appears to vary somewhat, whereas *hlyB* and *hlyC* are highly conserved. This suggests that there may be differences among hemolysins produced by different strains and, correspondingly, different levels of toxicity. Other observations indicate that the active site of all AH molecules are antigenically similar. The use of monoclonal antibodies might permit a more defined analysis of AH antigenicity and variability. A gene sequence has been identified which controls the level of hemolysin produced; studies now suggest that different levels of virulence relate, at least in part, to quantitative differences in hemolysin production.

Both plasmid and chromosomal hly determinants have flanking sequences identified as insertion sequences. The

presence of insertion sequences may explain the close homology between both plasmid and chromosomal hly determinants and the finding of identical hly determinants on plasmids of different incompatibility groups.

Most proteins in gram-negative bacteria usually require a signal peptide for transport through the bacterial inner membrane. They are then trapped in the periplasmic space or integrated into the outer membrane. Release from the cells can occur by several mechanisms. The *E. coli* hemolysin may be somewhat unique in that signal peptides have not been recognized. Instead processing, probably by proteolytic cleavage, is required for transport across the inner membrane. Specific proteins in the outer membrane, perhaps at limited sites, transport the hemolysin through the outer membrane and export hemolysin from the cell.

The data on chemical composition are unclear and confusing because of the use of different media for preparation of hemolysin and because of the lack of pure preparations. The chemical composition of AH preparations apparently is dependent upon the medium in which it is produced. All preparations studied thus far have contained protein, and hemolytic activity is destroyed by protease treatment. Lipid may be present in very minute quantities as suggested by inactivation with lipase. Carbohydrate has also been detected but only in AH produced in CDM. Whether AH is a protein, lipoprotein, or glycolipoprotein remains unclear and requires further research and better methods of purification and analysis. The molecular weight of AH is equally unclear. Estimates range from 58,000 to 580,000. This 10-fold variation might be explained by the suggested multimeric nature of AH or by the fact that AH may be complexed with various substances depending upon the type of medium in which it is produced. A hemolytically active protein has not yet been isolated in a homogeneous form from bacterial cultures. Consistent with the above, the stability of AH is also dependent upon the culture medium, the presence of divalent cations, and its relative state of purity. AH produced in meat infusion media is heat labile (56°C). In contrast, unpurified AH produced in CDM is heat stable in the absence of calcium ions but it becomes heat labile if purified or if calcium ions are added. Calcium is required for the hemolytic activity of AH produced in CDM but some reports indicate that it may not be required if the hemolysin is produced in alkaline meat extract broth. The role of calcium is unclear but it may induce a structural change in AH that leads to an active molecule. Clearly, the differences in the structure, stability, and calcium requirement of hemolysin produced in different media need to be resolved.

The mechanism and site of action are not yet known. However, the data suggest that the hemolysin may have a saponin-like effect and that the mechanism may be nonenzymatic. RBC exposed to AH undergo spherocyte formation and develop multiple small projections all over the membrane. A single hit of AH on an RBC is sufficient to cause lysis. How this occurs is unknown. The suggestion that AH may enter the cell and alter some biochemical process is bothersome if one accepts the data indicating a large molecular size. It would be of interest, if purified preparations were available, to label the molecule and determine whether there are binding sites on the membrane and whether the hemolysin translocates to the cytoplasm or cytoplasmic organelles.

Further research is needed to resolve the contradictions and to answer remaining questions regarding hemolytic *E. coli*. The biochemistry and molecular biology of AH have so far revealed a very complex and interesting phenomenon. Further clarification of the chemical and physical nature of the hemolysin and the mechanisms by which it affects host cells will require an intensive effort. New methods of isolation and the use of more sensitive methods for determining composition and structure will have to be used. Continuing efforts are needed to more clearly delineate the pathogenic mechanisms involved in the virulence of *E. coli* so that a better understanding of the biology of these organisms can be obtained.

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